ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2005, p. 431–433 Vol. 49, No. 1
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.1.431–433.2005

Interaction of Bismuth Subsalicylate with Fruit Juices, Ascorbic Acid, and Thiol-Containing Substrates To Produce Soluble Bismuth Products Active against *Clostridium difficile*

D. E. Mahony,[1]* A. Woods,[1] M. D. Eelman,[2] N. Burford,[2] and S. J. O. Veldhuyzen van Zanten[3]

Departments of Microbiology and Immunology,[1] Chemistry,[2] and Medicine,[3] Dalhousie University, Halifax, Nova Scotia, Canada

Received 12 May 2004/Returned for modification 31 August 2004/Accepted 25 September 2004

Bismuth subsalicylate (BSS), the active ingredient of Pepto-Bismol, has been used for many years to treat various disorders of the gastrointestinal tract. Using mass spectrometry and the agar dilution method, we determined that insoluble BSS interacts with certain dietary components and organic substrates to produce water-soluble products with activity against *Clostridium difficile*.

Clostridium difficile is a known cause of antibiotic-associated diarrhea and pseudomembranous enterocolitis. Although these conditions are usually responsive to antibiotic therapy with either metronidazole or vancomycin, some patients have frequent relapses (15) that are difficult to cure. In vitro studies have shown that *C. difficile* is sensitive to many synthetic bismuth compounds (1, 2, 3, 9, 10, 11, 13, 16) as well as to bismuth subsalicylate (BSS) (8) and colloidal bismuth subcitrate (13).

BSS, the active ingredient in Pepto-Bismol, is highly insoluble in water and yet reacts with substrates, such as cysteine (6, 14), to produce soluble bismuth products. Here, we show that BSS reacts not only with thiol-containing substrates but also with some common foods to produce soluble bismuth products and that these soluble bismuth products have excellent activities against *C. difficile*.

Soluble bismuth products were produced by adding 100 mg of BSS (Procter and Gamble, Cincinnati, Ohio) or 135 mg of $Bi(NO_3)_3$ (Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada) to 10 ml of each of the following fluids: water, 1% milk, ground coffee in water, orange juice, two commercial fruit drinks, freshly squeezed grapefruit and lemon juices, Australian red wine, V8 vegetable juice (Campbell Company of Canada, Toronto, Ontario, Canada), and fat-free chicken broth (Campbell). Similarly, BSS or $Bi(NO_3)_3$ was added to 200 mg of either L-(+)-cysteine hydrochloride monohydrate [L-(+)-cysteine-HCl] (EM Science, Gibbstown, N.J.), L-cysteine (Sigma-Aldrich), glutathione (Sigma-Aldrich), or DL-homocysteine (Sigma-Aldrich) dissolved in 10 ml of water and to 2 g of ascorbic acid (J. T. Baker Chemical Company, Phillipsburg, N.J.) in 10 ml of water. BSS was also added to four gastric juice samples obtained by endoscopy. These mixtures were stirred for 4 h at room temperature and filtered through a syringe filter (0.2-μm pore size). Insoluble bismuth salts did not pass through such a filter, as determined by inductively coupled mass spectrometry (ICPMS), and any bismuth contained in the filtrates was considered to represent soluble bismuth.

Serial twofold dilutions of the bismuth filtrates were made in sterile water and incorporated into Wilkins-Chalgren (WC) agar (Difco, Detroit, Mich.) to assess their antimicrobial activity. One milliliter of an overnight cooked meat culture of *C. difficile* (VPI 10463) was added to 10 ml of prereduced WC broth and incubated for 6 h in an anaerobic glove box (Forma Scientific, Marietta, Ohio). The average viable count of 14 such cultures was 3.2×10^7 CFU/ml. Serial 10-fold dilutions of the culture were made in WC broth, and duplicate 10-μl drops of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were inoculated onto the surfaces of the agar dilution plates. After anaerobic incubation for 24 h, the numbers of CFU were counted, and the MIC was defined as the concentration of elemental bismuth that completely inhibited the growth of a selected dilution of *C. difficile*. Inoculated duplicate and triplicate control plates contained no bismuth. A sample set of data used to determine MICs is presented in Table 1.

Filtrates of BSS in water demonstrated no activities against *C. difficile* and possessed virtually no detectable elemental bismuth; however, all thiol-containing substrates [L-cysteine, L-(+)-cysteine-HCl, glutathione, and DL-homocysteine] reacted with BSS to produce a water-soluble bismuth product. The pHs of the reaction mixtures ranged from 1 to 5. Acidification of an aqueous suspension of BSS did not solubilize BSS, and the gastric samples with a pH of 1 only marginally reacted with BSS to produce soluble bismuth products; therefore, the pHs of the mixtures did not seem to be responsible for rendering BSS soluble. Of the bismuth filtrates analyzed for elemental bismuth, the L-(+)-cysteine-HCl–BSS filtrate had the greatest concentration of soluble bismuth, and elemental analysis suggested that all of the insoluble bismuth was now accounted for as soluble bismuth. The interaction of BSS with thiol-containing molecules is not surprising, given the high affinity of the bismuth ion for sulfhydryl groups.

No antimicrobial activity was detected in filtrates obtained from the interaction of BSS with coffee, but there was moderate activity with milk and high activity with orange juice. With the exception of lemon juice, all the fruit juice-bismuth filtrates

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7. Phone: (902) 494-3888. Fax: (902) 494-5125. E-mail: David.E.Mahony@Dal.Ca.

TABLE 1. Data illustrating the determination of MICs for the soluble bismuth complexes formed by the interaction of BSS and L-(+)-cysteine-HCl

Reaction mixture	Dilution of bismuth complex	Bismuth concn (μg/ml)[a]	No. of CFU per indicated dilution of bacteria[b]		
			10^{-3}	10^{-4}	10^{-5}
Control (no bismuth)			TMC	37, 33	7, 7
			TMC	39, 34	5, 9
			TMC	39, 33	4, 1
BSS–L-(+)-cysteine-HCl[c]	1:320	20.32	0, 0	0, 0	0, 0
	1:640	10.16	0, 0	0, 0	0, 0
	1:1,280	5.08	0, 0	0, 0	0, 0
	1:2,560	2.54[d]	0, 0	0, 0	0, 0
	1:5,120	1.27	18, 19	2, 0	0, 0
	1:10,240	0.63	48, 43	4, 5	0, 1
	1:20,480	0.32	TMC	12, 19	1, 0

[a] Elemental bismuth was determined by ICPMS.
[b] A 6-h broth culture of *C. difficile* diluted in WC broth was used. Duplicate samples were counted on each plate. TMC, too many colonies to count.
[c] The reaction mixture, containing 100 mg of BSS, 200 mg of L-(+)-cysteine-HCl, and 10 ml of H_2O, was stirred for 4 h and filtered.
[d] The MIC in this experiment equals 2.54 μg/ml.

had high activities against *C. difficile*, especially the grapefruit juice filtrate. Grapefruit juice has received much attention because of its modifying effects on cytochrome P450 and the primary metabolism of certain drugs (4, 12). Our observations reflect another interaction of drugs with grapefruit juice, in which grapefruit juice reacts with BSS to produce soluble bismuth complexes. One assumes that this reaction would also take place in the gastrointestinal tract if grapefruit juice and BSS were concurrently consumed. Reactions of BSS with V8 juice and wine yielded lesser amounts of soluble bismuth product than those obtained with the fruit juices, and the yield from the reaction with the fat-free soup was almost negligible (Table 2).

Since a common ingredient in all of the active natural products might be vitamin C, we compared the reactions of BSS and $Bi(NO_3)_3$ with ascorbic acid. Both of these salts reacted similarly with ascorbic acid [and with L-(+)-cysteine-HCl] to produce soluble bismuth (Table 2). MIC determinations showed that ascorbic acid itself had activity against *C. difficile*, although the MIC was high (2,500 μg/ml); however, the MIC of the reaction product of BSS and ascorbic acid contained 156 μg of ascorbic acid/ml and 4.5 μg of elemental bismuth/ml. Since ascorbic acid does not contain sulfhydryl groups, the reaction of bismuth with ascorbic acid indicates the affinity of bismuth for other molecular functional groups.

TABLE 2. Elemental bismuth contents of soluble bismuth compounds and complexes formed by the interactions of BSS and bismuth nitrate with various food products, L-(+)-cysteine-HCl, or ascorbic acid

Reaction mixture tested	Elemental bismuth content of filtrate (μg/ml)[c]
BSS + water[a]	0
BSS + red wine[a]	140
BSS + squeezed grapefruit juice[a]	5,000
BSS + V8 juice[a]	520
BSS + soup[a]	3
$Bi(NO_3)_3$ + water[b]	190
BSS + L-(+)-cysteine-HCl[b]	5,700
BSS + ascorbic acid[b]	3,800
$Bi(NO_3)_3$ + L-(+)-cysteine-HCl[b]	5,500
$Bi(NO_3)_3$ + ascorbic acid[b]	4,300

[a] We used 100 mg of BSS and 10 ml of respective fluids, which were stirred for 4 h.
[b] We used 100 mg of BSS or 134 mg of $Bi(NO_3)_3$ and 200 mg of L-(+)-cysteine-HCl or 2 g of ascorbic acid and 10 ml of H_2O, which were stirred for 4 h.
[c] Determined by ICPMS.

Although the amount of insoluble bismuth converted to soluble bismuth differed depending upon the composition of the reaction mixture, the MICs of eight different reaction mixtures described in this communication were similar for each soluble bismuth product tested and ranged from 1.5 to 6.7 μg/ml (average, 3.8 μg/ml). The average MIC that inhibited by 10 to 100 times or more the number of bacteria was 7.1 μg/ml.

The presence of bismuth-cysteine complexes has been demonstrated previously by use of electrospray mass spectrometry (5, 6), but little is known about the fate of either soluble or insoluble bismuth medications when they interact with foods or specific amino acids. There have been several studies of the effects of BSS on diarrheal disease, but there is a paucity of information regarding *C. difficile*. Chang et al. (7) described an animal model with which the responses of *C. difficile* infection to vancomycin and BSS were compared. Vancomycin provided the best response; however, BSS treatment did result in delayed death for the animals.

In conclusion, we have shown that BSS interacts not only with molecules containing sulfhydryl groups, such as L-(+)-cysteine-HCl, DL-homocysteine, and glutathione, but also with fruit juices and ascorbic acid. Our less intensive studies with $Bi(NO_3)_3$ indicated similar interactions. We have demonstrated that the reaction products described have very good activities against *C. difficile*, a major pathogen of the colon. Whether they have therapeutic potential must be assessed, but Pepto-Bismol itself deserves further assessment for its potential to cure *C. difficile* infections in vivo.

We thank the Procter and Gamble Corporation for supplying the BSS used in these experiments. We also acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada and the Killam Program of the Canada Council for the Arts.

REFERENCES

1. **Agocs, L., N. Burford, T. S. Cameron, J. M. Curtis, J. F. Richardson, K. N. Robertson, and G. B. Yhard.** 1996. Spectroscopic, structural, and mass spectrometric studies on two systematic series of dithiabismuth(III) heterocycles: identification of bismuthenium cations and their solvent complexes. J. Am. Chem. Soc. **118:**3225–3232.
2. **Agocs, L., G. G. Briand, N. Burford, T. S. Cameron, W. W. Kwiatkowski, and K. N. Robertson.** 1997. The structurally flexible bicyclic bis(2-hydroxyethanethiolato)bismuth(III) complex: a model for asymmetric monoanionic chelation of bismuth(III). Inorg. Chem. **36:**2855–2860.
3. **Asato, R., K. Kamamuta, Y. Akamine, T. Fukami, R. Nukada, M. Mikuriya, S. Deguchi, and Y. Yokota.** 1997. Bismuth (III) complexes of 2-mercaptoethanol: preparation, structural and spectroscopic characterization, antibacterial activity toward *Helicobacter pylori*, and inhibitory effect toward *H. pylori*-produced urease. Bull. Chem. Soc. Jpn. **70:**639–648.
4. **Bailey, D. G., M. Arnold, and D. Spence.** 1998. Grapefruit juice-drug interactions. Br. J. Clin. Pharmacol. **46:**101–111.
5. **Burford, N., M. D. Eelman, and T. S. Cameron.** 2002. Comprehensive characterization of bismuth thiosalicylate complexes: models for bismuth subsalicylate. Chem. Commun. (J. Chem. Soc. Sect. D) **2002:**1402–1403.
6. **Burford, N., M. D. Eelman, D. E. Mahony, and M. Morash.** 2003. Definitive identification of cysteine and glutathione complexes of bismuth by mass spectrometry: assessing the biochemical fate of pharmaceutical agents. Chem. Commun. (J. Chem. Soc. Sect. D) **2003:**146–147.

7. **Chang, T.-W., M.-Y. Dong, and S. L. Gorbach.** 1990. Effect of bismuth subsalicylate on *Clostridium difficile* colitis in hamsters. Rev. Infect. Dis. **12**(Suppl. 1)**:**S57–S58.
8. **Cornick, N. A., M. Silva, and S. L. Gorbach.** 1990. In vitro antibacterial activity of bismuth subsalicylate. Rev. Infect. Dis. **12**(Suppl. 1)**:**S9–S10.
9. **Diemer, R., B. K. Keppler, U. Dittes, B. Nuber, V. Seifried, and W. Opferkuch.** 1995. Preparation and characterization of biologically active bismuth (III) tropolonato complexes. Chem. Ber. **128:**335–342.
10. **Dittes, U., E. Vogel, and B. K. Keppler.** 1997. Overview on bismuth (III) and bismuth (V) complexes with activity against *Helicobacter pylori*. Coord. Chem. Rev. **163:**345–364.
11. **Domenico, P., R. J. Salo, S. G. Novick, P. E. Schoch, K. Van Horn, and B. A. Cunha.** 1997. Enhancement of bismuth antibacterial activity with lipophilic thiol chelators. Antimicrob. Agents Chemother. **41:**1697–1703.
12. **Fuhr, U.** 1998. Drug interactions with grapefruit juice. Extent, probable mechanism and clinical relevance. Drug Saf. **18:**251–272.
13. **Mahony, D. E., S. Lim-Morrison, L. Bryden, G. Faulkner, P. S. Hoffman, L. Agocs, G. G. Briand, N. Burford, and H. Maguire.** 1999. Antimicrobial activities of synthetic bismuth compounds against *Clostridium difficile*. Antimicrob. Agents Chemother. **43:**582–588.
14. **Sadler, P. J., H. Sun, and H. Li.** 1996. Bismuth (III) complexes of the tripeptide glutathione (γ-L-Glu-L-Cys-Gly). Chem. Eur. J. **2:**701–708.
15. **Settle, C. D., and M. H. Wilcox.** 1996. Antibiotic-induced *Clostridium difficile* infection. Aliment. Pharmacol. Ther. **10:**835–841.
16. **Summers, S. P., K. A. Abboud, S. R. Farrah, and G. J. Palenik.** 1994. Syntheses and structures of bismuth (III) complexes with nitrilotriacetic acid, ethylenediaminetetraacetic acid, and diethylenetriaminepentaacetic acid. Inorg. Chem. **33:**88–92.